T and NK cell immunity after hematopoietic stem cell transplantation

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Chapter 1

General introduction

1 Allogeneic hematopoietic stem cell transplantation

Hematopoietic stem cells constitute a life-time source for red blood cells and platelets as well as for white blood cells that form the immune system. The allogeneic transplantation of these stem cells provides a therapy for severe hematological, immunological and metabolic disorders by replacing the patients (defect) hematopoietic system with that of a healthy donor.[1] Furthermore, hematopoietic stem cell transplantation (HSCT) is applied as rescue therapy in patients with hematological malignancies, to restore the hematopoietic system after intensive chemo- and radiation therapy which not only kills leukemic cells but also kills healthy hematopoietic stem cells.[1]

1.1 History

In 1968, both in Minneapolis and Leiden, the first successful allogeneic HSCT were performed with HLA-identical sibling donors in children suffering from severe combined immune deficiency (SCID).[2-4] In the last 50 years, the worldwide number of allogeneic HSCT that is performed annually has grown exponentially to almost 16.000 in 2014 (Figure 1.1).[5] After the first transplantation for SCID, HSCT was also successfully used to treat severe aplastic anemia (SAA) and as rescue therapy for hematological malignancies.[4;6;7] The first decades, HSCT was marked by a high treatment related morbidity and mortality.[1] When outcomes of HSCT improved as result of advances in transplantation technology, supportive care and improved HLA typing, HSCT became an acceptable treatment option for severe hemoglobinopathies as well as severe metabolic disorders.[8] In the early years, the applicability of HSCT was limited to the use of HLA identical sibling donors (identical related donor, IRD). The first successful HLA matched unrelated donor (MUD) transplantation was performed in 1979, facilitating allogeneic HSCT for patients with an HSCT indication for whom no IRD was available.[9] In the next decade, haplo-identical donors and cord blood (CB) transplantations were introduced as alternatives for IRD and MUD transplantations.[10-12] Whereas bone marrow (BM) was the main stem cell source in the first 25 years of HSCT, the use of stem cells that are mobilized into peripheral blood (PBSC) has grown extensively since the introduction in 1993.[5;13]

Over the years, scientific developments have greatly improved the outcome after HSCT (Figure 1.1). The morbidity and mortality after MUD transplantations are now comparable to the IRD setting, while the outcome of HSCT in patients who received an alternative donor graft (CB or haplo-identical) is steadily improving and approaching the outcome in MUD transplantations. This is mainly due to the increasing understanding of transplant immunology, technical developments in HLA-typing, lower toxicity of conditioning regimens, ongoing improvements in the procedures to reduce alloreactivity and infectious complications and, more recently, also personalized medicine, such as therapeutic drug monitoring and personalized conditioning.

The successful outcome after HSCT depends on the selection of the best donor and graft source and the well-considered and targeted application of strategies to reduce the risk of graft-versus host disease without compromising immune reconstitution. This balance will be further discussed in the next sections.

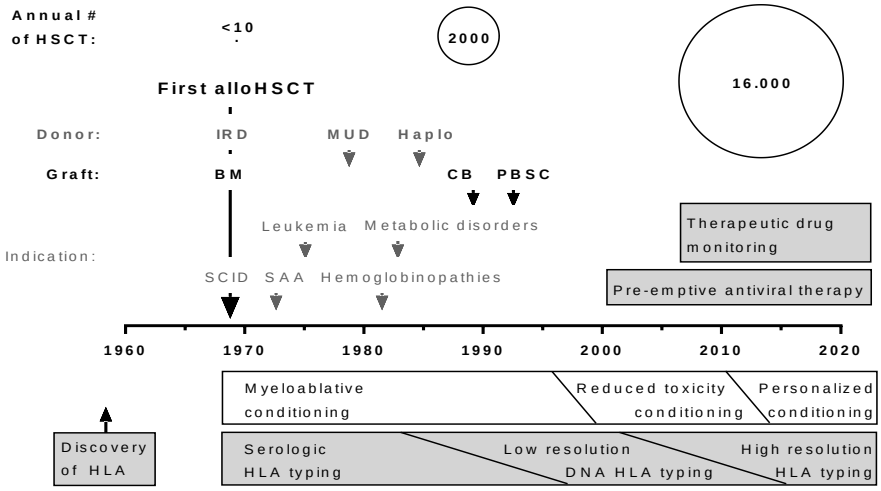

Figure 1.1 Milestones and major improvements in the history of allogeneic HSCT.

1.2 Alloreactivity

The largest challenge for the successful application of allogeneic HSCT is the prevention and management of alloreactivity. Alloreactivity is caused by T cells recognizing histo-incompatible cells. The chance of alloreactivity is increased when HLA-disparity is present between donor and recipient, but alloreactivity can also be directed to minor histocompatibility antigens.[14-16]

Host-versus-graft reactions are caused by recipient T cells and lead to the killing of donor (stem) cells leading to failure of engraftment or graft rejection, which is associated with high mortality due to secondary infections.[17]

Graft-versus-host reactions are caused by donor T cells and are directed to both hematopoietic and non-hematopoietic tissues of the recipient. Graft-versus-host reactions directed to hematopoietic cells of the recipient can be beneficial as the killing of recipient cells in the bone marrow creates space for the donor stem cells. Also, leukemic cells that survived the conditioning therapy can be killed by donor T cells in a so-called ***graft-versus-leukemia*** (GvL) reaction.[15]

However, graft-versus-host reactions also cause ***graft-versus-host disease*** (GvHD). The pathophysiology of GvHD can be described in three steps: first, the conditioning therapy causes tissue damage and inflammation, leading to activation of antigen presenting cells (APC) of the host. Second, donor-derived cells interact with these host APC to get activated and expand. Thirdly, donor T cells cause further tissue damage and a general inflammatory condition, which initiates a vicious circle.[16] GvHD can be both acute and chronic, and tissues mainly affected are skin, intestine and liver. Symptoms can vary from a skin rash to desquamation, and from nausea to massive bloody diarrhea and ileus. In case of (suspected) GvHD, treatment is installed using immunosuppressive medication, starting with high dose steroids. As second line treatment, other immunosuppressive drugs and biologicals (e.g. MMF and Infliximab) are used.[16] In an experimental setting, extracorporal photopheresis and the infusion of mesenchymal stromal cells

are applied. Steroid refractory GvHD is associated with a high mortality, both directly and because of immunosuppression and secondary infections.[16]

1.3 HLA-matching, donor type and graft source

The first step in the prevention of alloreactivity is the search for an optimal, HLA-matched donor (Figure 1.2). The human HLA system is highly complex and polymorphic. It consists of 12 genes on the short arm of chromosome 6 that encode for HLA class I antigens (HLA-A, -B and -C) and HLA class II antigens (DR, DQ, DP). Currently, over 2500 HLA-alleles have been identified, resulting in an immense possibility of unique combinations. At first, HLA typing was based on serologic (antigenic) testing (*e.g.* HLA-A2) or low resolution molecular (DNA) typing (*e.g.* A*02). Because each serotype contains different alleles, a donor matched at the serotype level can still be mismatched at the allele level. Therefore, high resolution molecular typing at the allele level is nowadays used to match unrelated donors (*e.g.* A*0201). The 5 or 6 most important HLA genes (A/B/C/DRB1/DQB1(/DPB1)) are routinely tested in the matching procedure before HSCT. Because genes encoding these major HLA alleles are present on both chromosomes, a full HLA match is 10/10 or 12/12.[14;18]

An ***HLA identical related donor*** (IRD) is the donor of first choice. Due to Mendelian inheritance, the chance that two siblings are HLA identical is 25%. An IRD is available in 13-50% of patients, which is dependent on the number of siblings.[19;20]

If no IRD is available, the search is extended, looking for a ***matched unrelated donor*** (MUD) in the Bone Marrow Donors Worldwide database.[21] MUD donors are available for 30-70% of HSCT recipients.[19;20] The chance a MUD is available is largely dependent of the ethnic background of the recipient. Caucasians have a better chance of finding a MUD than people with an ethnic minority background[20;22]. If no fully matched MUD donor is available, the best matched unrelated donor (*e.g.* 9/10 or 8/10 match) can be used, at the cost of an increased risk of alloreactivity.

Alternatives for MUD and IRD transplantation are haplo-identical donors or cord blood (CB) transplantation.[23] In contrast to IRD and MUD donors, both cord blood units and haplo-identical donors are almost always directly available. Although for different reasons, both haplo-identical and cord blood transplantations are associated with a delayed immune reconstitution and increased risk for infectious complications in comparison to IRD and MUD transplantations.[1;4]

Haplo-identical donors are family members (*e.g.* parents or children) that share one haplotype with the recipient, resulting in a 5/10 HLA match.[23] Due to the high level of HLA-disparity, haplo-identical transplantations require additional strategies to reduce the risk of graft rejection and GvHD, which may also cause a delayed immune reconstitution, and consequently increase the risk of infectious complications. However, the outcome of haplo-identical transplantation has greatly improved over time as a result of ongoing improvements in the procedures applied to reduce alloreactivity and infectious complications, which will be discussed later.[23]

Cord blood is a residual product that naturally contains a high concentration of hematopoietic stem cells which is cryopreserved and stored in cord blood banks.[24;25] Cord blood grafts are rapidly available, have a high regenerative capacity and are relatively tolerant, allowing for larger HLA mismatches.[25] However, the rate of immune reconstitution after CB transplantation is

highly dependent on the total number of stem cells that is present in a cord blood graft, carrying the risk of delayed immune reconstitution and infectious complications.[24;25] To improve immune reconstitution, double cord blood units can be given. Due to the advances in HLA-matching technologies and increasing numbers of available CB units, the matching of CB transplantations has improved.[25] Together with advances in the procedures to reduce alloreactivity, this has led to strong improvements in immune reconstitution and the outcome of CB transplantations.[24;25]

The neonatal period is the only moment that hematopoietic stem cells circulate in the blood at high concentrations. For adult donors, hematopoietic stem cells can be harvested from ***bone marrow*** (BM) or ***peripheral blood*** **(**PBSC) after mobilization with granulocyte colony-stimulating factor (G-CSF) mobilization.[24-26] The advantage of PBSC donation is that no general anesthesia is needed for the donor. Because PBSC grafts contain 10 times more lymphocytes than BM grafts, the immune reconstitution after PBSC transplantation is often faster than after BM transplantation. However, PBSC grafts are associated with an increased risk of chronic GvHD.[26] For pediatric donors, BM donation is preferred to limit the exposure of healthy children to G-CSF.

1.4 Conditioning therapy

The HSCT starts with the preparation of the recipient during conditioning therapy (Figure 1.2). The conditioning therapy is given in the weeks before HSCT. It aims to eradicate the recipient's hematopoietic stem cells and ***makes space in the stem cell niche*** of the bone marrow (myeloablation). The stem cell niche in bone marrow is required to support the infused donor stem cells from the graft to proliferate and differentiate. Secondly, the conditioning therapy ***eradicates residual malignant cells*** to improve the chance of leukemia free survival. Thirdly, the conditioning therapy ***eliminates the recipient's immune system*** to prevent the rejection of the graft by the recipients T cells (immunoablation).[1;27] Many different conditioning regimens have been developed and can be divided in myeloablative or non-myeloablative regimens. In general, myeloablative regimens consist of an alkylating agent (*e.g.* Busulfan, Melphalan, Treosulfan) or Total Body Irradiation (TBI), in combination with an immunoablative agent (*e.g.* Fludarabine).[27]

Non-myeloablative or reduced intensity conditioning regimens consist of a low dose of an alkylating agent or TBI, in combination with an immunoablative agent. Non-myeloablative regimens are mainly applied in older patients and patients with a poor clinical condition to reduce organ toxicity.[1;27]

In the last decades, the conditioning therapy has greatly improved, resulting in less toxic, more effective conditioning regimens. For example, TBI is frequently replaced by Busulfan and, more recently, Treosulfan. Also, Cyclophosphamide is increasingly being replaced by Fludarabine in most (pediatric) HSCT regimens.[27] In addition, therapeutic drug monitoring and personalized dosing regimens contributed to a reduction of toxicity.[28]

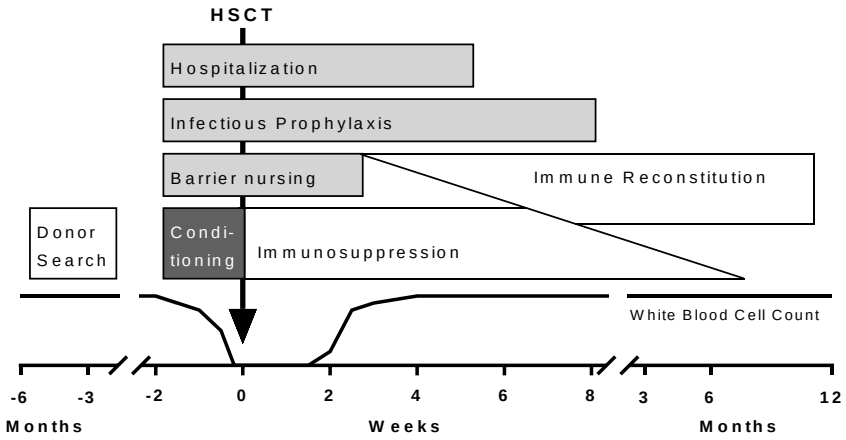

Figure 1.2. Overview of the allogeneic HSCT procedure.

1.5 Prevention of graft-versus-host disease

The risk of GvHD is the product of the level of HLA-matching, donor type and graft source, and is strongly dependent on T cells co-transplanted with the graft. Based on these input parameters, the best combination of strategies to prevent alloreactivity is chosen. Interventions to reduce the risk of graft-versus-host disease, which will be discussed in this paragraph, include graft manipulation, serotherapy and post-transplant immunosuppression.

Too strong suppression of alloreactivity, however, may lead to reduction of graft-versus-leukemia reactions as well as delayed immune reconstitution, increasing the risk of infectious complications. Therefore, interventions to reduce the risk of GvHD have to be applied well-considered and targeted (Figure 1.3).

Besides hematopoietic stem cells, the stem cell graft also contains lymphocytes. Co-transplanted T cells can have both positive (*e.g.* faster immune reconstitution) and negative effects (*e.g.* risk of GvHD). ***Graft manipulation*** can be used to reduce the number of T cells that are present in the graft. Both the selection of hematopoietic stem cells (positive selection) and depletion of T cells (negative selection) are applied for this purpose. This reduces the risk of GvHD, but is associated with delayed T cell reconstitution. Graft manipulation is mainly used for mismatched unrelated and haplo-identical donor transplantations.[23;24]

Serotherapy is the treatment with lymphocyte depleting antibodies usually administered to the patient in the week before the HSCT, with the aim to reduce the risk of graft rejection and GvHD. Only IRD transplant recipients do not routinely receive serotherapy. Serotherapy is a special form of immuno-ablative conditioning therapy. Due to the long half-time of the antibodies, they are still present during the HSCT procedure and in the weeks thereafter. Consequently, not only the recipients T cells are killed (preventing graft rejection), but also donor T cells that are present in the graft and donor T cells that reconstitute from the graft in the early period after engraftment are killed. Serotherapy plays an important role in the prevention of GvHD, but overdosing of serotherapy has a negative impact on immune reconstitution.[29;30] Different serotherapy agents are

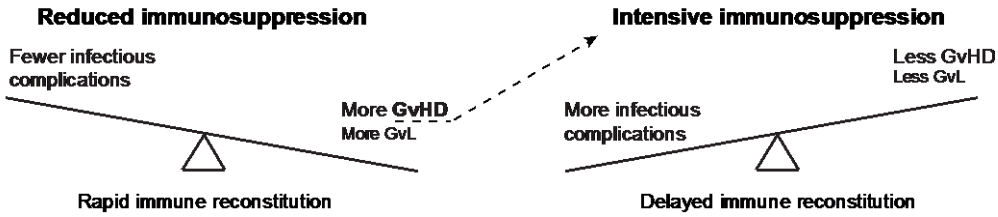

Figure 1.3. Balance between the risk of GvHD and infectious complications after HSCT.
The risk of GvHD and infections are related to interventions to prevent alloreactivity, such as T cell depletion of the graft, serotherapy before HSCT and immunosuppressive drugs after HSCT. Abbreviations: GvHD: Graft versus host disease, GvL: graft versus leukemia reaction.

used in the HSCT setting. Anti-thymocyte globulin (ATG) is a polyclonal antibody preparate, produced in rabbits or horses after immunization with human thymocytes or human T cell lines. ATG recognizes a wide range of antigens present on lymphocytes, but also other tissues like endothelial cells. Alemtuzumab (Campath®) is a humanized monoclonal antibody recognizing CD52, an antigen present on all lymphocytes. The half-life of ATG and Alemtuzumab is 4-14 days and 15-21 days, respectively.[31;32] Consequently, immune reconstitution is slower in Alemtuzumab treated patients.[33]

In the recent years, retrospective pharmacokinetics/pharmacodynamics studies have generated knowledge to enable the personalized dosing of serotherapy based on body weight and the number of lymphocytes present at the start of condition.[30] In the near future, personalized dosing of serotherapy may prevent overdosing of serotherapy, resulting in faster T cell reconstitution without an increased risk of GvHD.[30]

Post-transplant immunosuppressive drugs are used routinely to prevent GvHD. These drugs prevent the activation of proliferation of activated (alloreactive) T cells.[16] Often, the calcineurin inhibitor Cyclosporin A is used either alone or in combination with a short course of Methotrexate. Depending on the HLA-matching, donor type and graft source, also Mycophenolate Mofetil, Tacrolimus, Sirolimus and prednisone are sometimes used for the prophylaxis of GvHD.[16] The duration of post-transplant immunosuppression depends on the indication for the HSCT. In patients with hematological malignancies, immunosuppressive drugs are often tapered after 6-8 weeks if GvHD does not occur, to stimulate the graft-versus leukemia reactions.[34] For patients with non-malignant HSCT indications, for whom alloreactivity is not beneficial and should be avoided, immunosuppressive drugs are generally tapered after 3-6 months (Figure 1.2 and 1.3).[16]

Besides the major determinants HLA-matching, donor type and graft source, also genetic factors (*e.g.* polymorphisms of cytokines and/or -receptors), conditioning-induced tissue-damage and the composition of the intestinal microbiome are associated with the development of GvHD. Therefore, ***reduced-toxicity conditioning*** and the use of ***intestinal decontamination*** to eliminate the microbiome can also reduce the risk of GvHD.[16;35;36]

2. Viral complications after HSCT

T cell immunity is pivotal to provide protection against and achieve sustained control of viral infections.[37-39] In immunocompetent individuals, herpesvirus infections generally cause mild symptoms after which latency is established and reactivations can occur sporadically throughout life.[40] During the T cell deficient period after HSCT, patients are at risk for the reactivation of latent herpesvirus infections as well as a protracted and severe course of respiratory virus infections. From the group of herpesviruses, cytomegalovirus (CMV) and Epstein-Barr virus (EBV) reactivations have the largest clinical impact, whereas human adenovirus (HAdV) is responsible for the most severe respiratory and gastro-intestinal complications in pediatric HSCT recpients.[41-44]

Viral complications occur mainly in HSCT recipients at risk for a delayed immune reconstitution, such as patients who received (a high dose of) serotherapy (Figure 1.4, patient 2 and 3).

Three groups of patients have an increased risk of severely delayed T cell reconstitution leading to disseminated and life-threatening virus infections [41-44] (Figure 1.4, patient 3):

1. haplo-identical transplantation with T cell depletion of the graft,
2. cord blood transplantations with high dose serotherapy and a delayed immune reconstitution, *and*
3. severe immunosuppression due to graft-versus host disease.

The prevention of viral reactivation and infection would be the best way to reduce morbidity and mortality caused by these viruses. The prophylactic use of antiviral medication is restricted by the limited effectiveness and considerable toxicity of antiviral drugs. For CMV, EBV and HAdV, no effective prophylactic drugs are available. Once these viruses become symptomatic, they are very often disseminated and treatment started at that moment will be too late. For this reason, these viruses are routinely monitored in high risk patients.[45] Monitoring is performed on weekly plasma samples in which viral load is determined by polymerase chain reaction (PCR) technology. Once the viral load reaches a certain threshold (most often 1000 DNA copies/ml of blood) at two consecutive time points, pre-emptive treatment is installed.[45]

2.1 Cytomegalovirus and Epstein Barr virus

For both CMV and EBV, primary infection in young children causes mild flu-like symptoms, whereas primary infection in adolescents and adults can cause infectious mononucleosis (M. Pfeiffer, EBV) or infectious mononucleosis-like disease (CMV).[40] In the adult population, 60% and 90% of individuals is seropositive for CMV and EBV, respectively. As a consequence, most HSCT recipients are at risk for reactivation. Seropositive patients, who receive a graft from a seronegative donor, are at risk for severe infections as no virus-specific memory T cells are co-infused with the graft.[42;43;46] The selection of donors-recipient combinations based on CMV serostatus is applied, but this is only possible when two or more donors with a comparable HLA match are available.[46]

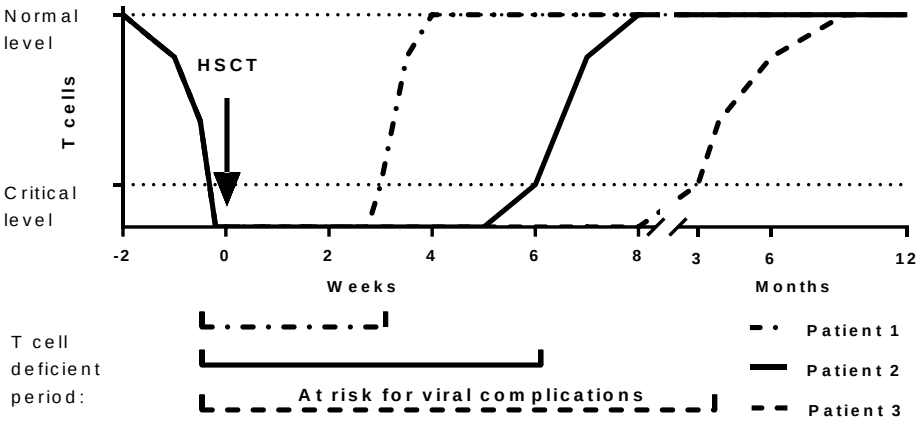

Figure 1.4 Relation between T cell reconstitution and the risk off viral complications.
Patient 1: IRD transplantation, no serotherapy. Patient 2: MUD transplantation with serotherapy. Patient 3: Cord blood transplantation with serotherapy or haplo-identical transplantation with T cell depletion and serotherapy.

CMV reactivation after HSCT can lead to disseminated infections causing interstitial pneumonitis, colitis and hepatitis.[43] EBV reactivation in immunocompromised patients can lead to post-transplant lymphoproliferative disease (PTLD), a life-threatening neoplastic condition caused by the uncontrolled proliferation of EBV-infected B cells.[42]

For CMV, pre-emptive treatment consists of intravenous ganciclovir or foscarnet. Since the introduction of pre-emptive CMV treatment, two decades ago, morbidity and mortality have dropped significantly. However, both drugs have a considerable toxicity profile. Ganciclovir causes myelosuppression, which is undesirable after HSCT, and foscarnet is associated with severe nefrotoxicity.[47]

For EBV, rituximab, a monoclonal antibody against CD20, is used as pre-emptive treatment. Rituximab depletes B cells, thereby removing the major pool of cells the virus is dependent on for its replication. The use of rituximab has led to a strong reduction of EBV-PTLD, but is obviously associated with a delayed B cell reconstitution.[39;42]

2.2 Other herpesviruses

Herpes Simplex Virus (HSV) and Varicella Zoster Virus (VZV) reactivations after HSCT can have a prolonged course but are often limited to oral mucosa and skin. Dissemination of disease, causing pneumonia or meningitis sporadically occurs.[48] For HSV and VZV, prophylactic acyclovir treatment is applied successfully.[48] Human herpesvirus 6 (HHV6) causes the sixth disease (exanthema subitum) upon primary infection. Reactivations generally occur in the first month after HSCT and cause fever and skin rash, which is generally self-limiting but may be mistaken for GvHD.[49]

2.3 Human Adenovirus

Although HAdV infection does not lead to a state of latency, the virus can remain detectable for a substantial period of time after primary infection. This may be the reason that HAdV infections or reactivations are mainly seen in pediatric HSCT recipients. Currently, more than 50 serotypes have been described. In healthy individuals, HAdV infections cause self-limiting infections such as conjunctivitis, upper respiratory tract-, urinary tract- or gastrointestinal infections.[50] After HSCT, HAdV reactivations or primary infections can progress to viremia and disseminated disease. In the absence of T cell surveillance, the mortality of HAdV viremia is high because of progression to HAdV related multi-organ failure.[41;51]

Ribavirin and cidofovir have been explored for the pre-emptive treatment of HAdV infections. However, HAdV remains a major clinical problem despite pre-emptive treatment. Ribavirin was shown to be ineffective in patients without lymphocyte reconstitution.[52] Cidofovir is commonly used as pre-emptive treatment for HAdV but is associated with severe nephrotoxicity.[53-56] Also, the clinical effect is often disappointing, with HAdV clearance ranging from 24-98% of patients.[54-59] Recently, promising data have been published for brincidofovir, the orally bioavailable lipid conjugate of cidofovir. This drug has a reduced toxicity profile, while higher intracellular concentrations are reached. The majority of HSCT recipients with HAdV infections had a reduction of HAdV load during brincidofovir treatment.[60;61]

3. Immune reconstitution after HSCT

As discussed in the previous sections, immuno-ablation, leading to white blood cell aplasia, is applied in order to create a permissive environment for the stem cell graft and prevent alloreactivity. However, this induced immunodeficiency is associated with an increased risk for opportunistic infections (Figure 1.3). During white blood cell aplasia, patients are at risk for severe bacterial, viral and fungal infections. A disseminated course of viral and fungal infections may occur during lymphopenia. Therefore, the rapid reconstitution of the immune system is of great importance to reduce transplant related morbidity and mortality.

In this section, the typical pattern of immune reconstitution will be described, as well as the main factors influencing immune reconstitution and interventions explored to improve immune reconstitution. In view of the other chapters in this thesis, the focus of this section will be on T cells and NK cells.

Generally, the cells of the innate immune system (Monocytes, Neutrophils and NK cells) recover within a month after HSCT. T cell reconstitution often occurs in the second month whereas B cells are the last lymphocyte subset that reconstitutes after HSCT (Figure 1.5).[33;62] As already discussed in the first section of this chapter, many HSCT related parameters have an impact on immune reconstitution. Especially T cell reconstitution is strongly affected by T cell depletion of the graft and serotherapy (Figure 1.4).

3.1 Recovery of innate immunity

The first line of immunity is represented by the epithelial and mucosal barriers that prevent tissues against invasion of microbes. This barrier is often disrupted by the pretransplant chemotherapy and irradiation and usually recovers within weeks, although this can be delayed by GvHD.[63] Most complement factors are produced by liver cells and are generally not deficient after HSCT.[64;65] Neutrophils and monocytes are the first cells that recover from the donor derived stem cells.

Neutrophil recovery is used as a marker for engraftment, defined by the moment neutrophils reach 0.5×10^9 cells / ml of blood.

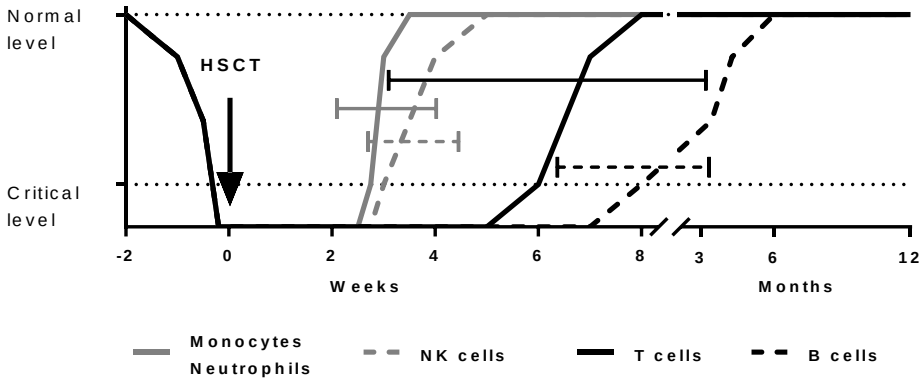

Figure 1.5. Typical reconstitution of innate (gray) and adaptive (black) immune cell subsets after HSCT.

Engraftment generally occurs within 2-4 weeks after HSCT (Figure 1.5).[33;62] Rapid engraftment is observed in patients who received a PBSC graft and delayed engraftment may occur in patients who received a cord blood graft or a graft from a haplo-identical donor. Dendritic cells in blood reconstitute from donor stem cells within weeks after HSCT. However, the replacement of the recipients tissue-residing antigen presenting cells may take several months.[66;67] Natural killer (NK) cells are the first lymphocytes that reappear in the circulation after HSCT (Figure 1.5). The reconstitution of NK cells will be discussed separately in the last section of this chapter.

3.2. T lymphocytes

T cells are the key players of the adaptive immune system and provide life-long pathogen specific protection. As a result of T cell receptor rearrangement, each T cell clone recognizes only one specific epitope. T cells develop and are educated in the thymus. In the thymus, T cells that do not recognize self-MHC and T cells that have a too strong affinity to self-MHC are removed to end up with useful T cells that can interact with other cells, but which are generally not auto-reactive (central tolerance).[68] However, T cells are only educated to be tolerant for self-MHC, leaving the possibility of occurrence of alloreactivity after HSCT. The human T cell compartment consists of $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells. $CD4^+$ T cells orchestrate immune responses via the secretion of cytokines and interaction with other immune cells. Distinct subpopulations of $CD4^+$ T cells have specific cytokine profiles to direct (*e.g.* Th1, Th2 and Th17) or regulate (regulatory T cells, Treg) the immune response.[69] $CD8^+$ T cells are cytotoxic and destroy virus-infected and tumor-transformed cells. Once naïve T cells have encountered their specific antigen, T cells are activated, proliferate and differentiate into effector and memory cells. The differentiation of human T cells can be discerned based on the expression of cell surface markers (*e.g.* CD45RA and CCR7, Figure 1.6). Naive ($CD45RA^+$ $CCR7^+$) cells differentiate upon antigen exposure into central memory (CM, $CD45RA^-$ $CCR7^+$) and effector memory (EM, $CD45RA^-$ $CCR7^-$) cells and eventually regain CD45RA when differentiating into end-stage effector (EMRA, $CCR7^-$ $CD45RA^+$) cells.[70] Whereas the expression of co-stimulatory molecules and chemokine receptors varies between the differentiation stages, ex-vivo cytolytic capacity increases during differentiation and telomere length shortens.[70]

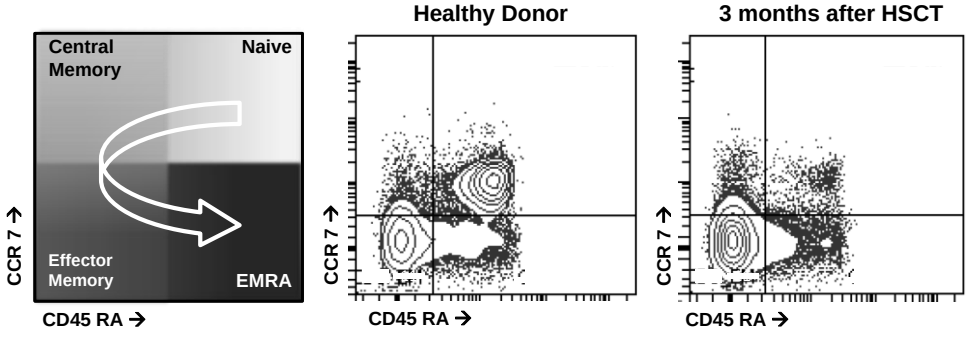

Figure 1.6. T cell differentiation stages.
T cell differentiation can be discerned based on the expression of CCR7 and CD45RA in healthy individuals and early after HSCT. $CD8^+$ T cell differentiation is depicted.

3.2.1 T cell reconstitution after HSCT

The recovery of normal numbers of T lymphocytes after HSCT takes months, but the complete and balanced reconstitution of the adaptive immune system ranging from naïve cells to effector- and memory cells takes many years.[62;71]

In the first months after HSCT, the repopulation of the T cell compartment is facilitated by cytokine- and antigen-driven homeostatic peripheral expansion of T cells that were transplanted with the graft. Both memory cells and naïve cells that were transplanted with the graft will differentiate and expand rapidly when they encounter the antigen of their specificity. As a result, the first stage of T cell reconstitution is strongly skewed towards memory cells (Figure 1.6).[33;62] This expansion of memory cells is more rapid for $CD8^+$ T cells than for $CD4^+$ T cells. The early reconstitution of T cells is strongly affected by T cells depletion of the graft, as well as the use of serotherapy (Figure 1.3). Also, cord-blood grafts are associated with a delayed T cell reconstitution.[33;62]

The thymus-dependent generation of naive T cells, essential for building a balanced immune system with a broad specificity against neo- and recall antigens is delayed for many months to years.[71] T cells that develop from donor stem cells will undergo selection in the thymus of the recipient and should be tolerant for the recipient's tissues. In children, the reconstitution of naïve T cells is better than in adults, which is related to the involution of the thymus during life. Damage to the thymus caused by the conditioning regimen or GvHD has a negative impact on the reconstitution of naïve T cells.[62;71]

3.2.2 Strategies to improve T cell reconstitution

Reconstitution of $CD4^+$ and $CD8^+$ T cell immunity is pivotal to provide protection against and achieve sustained control of viral reactivations as well as anti-leukemic reactions.[15;37-39] However, T cells are also responsible for GvHD, which is mainly caused by (naïve) T cells transplanted with the graft.[16] The improvement of T cell reconstitution is therefore a delicate issue. The easiest way to improve T cell reconstitution is the omission of serotherapy from the conditioning regimen, as is applied in the IRD transplant setting. However, this is not possible in other transplant settings due to the risk of GvHD. However, personalized dosing of serotherapy can be applied to prevent overdosing and results in faster T cell reconstitution without increasing the risk of GvHD.[30] Also, the early tapering of CsA immunosuppression and omission of methotrexate from GvHD prophylaxis has been used to improve T cell reconstitution and to enable GvL reactions.[34]

Alternatively, donor lymphocytes can be infused in the period after the HSCT. Donor lymphocyte infusions (DLI) contain both virus-specific memory T cells as well as naïve T cells that can generate de novo graft-versus-leukemia reactions.[72;73] Even when given months after HSCT, under non-inflammatory conditions, unselected DLI are still associated with the development of GvHD.[72;73] The use of unselected DLI in the early, inflammatory phase after HSCT to treat viral infections bears a large risk of GvHD development. Therefore, adoptive transfer of virus specific T cells from the donor to restore anti-viral immunity after HSCT has been explored. By the *ex vivo* selection of virus-specific cells, co-transfer of alloreactive cells can be limited, thus reducing

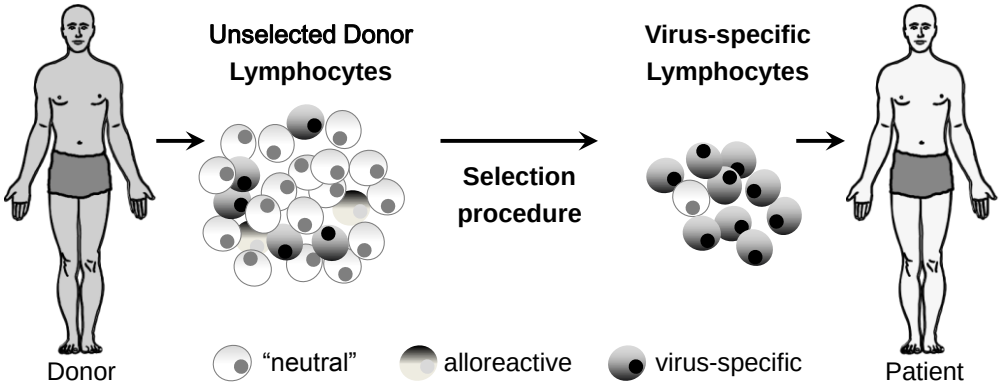

Figure 1.7. Selection of virus-specific T cells for adoptive immunotherapy.

the risk of GvHD development (Figure 1.7). The first approach used for the generation of virus-specific T cell products is the repetitive stimulation of donor lymphocytes with APCs transfected with viral vectors encoding for viral peptides (CMV, HAdV) or EBV-transformed B cells. This strategy yields large numbers of virus-specific (mainly $CD8^+$) T cells which have been safely used in clinical trials.[74-76] However, due to the high workload and long culture period, this approach is unfeasible for broad application. The second approach aims to select virus-specific cells directly *ex vivo*. Tetramer based magnetic isolation uses MHC class I tetramers loaded with viral peptides, which bind virus-specific cells via their T cell receptor. The virus-specific cells are then labelled with magnetic beads, and isolated via a magnetic column. This method is rapid and has been used for the selection of CMV-specific $CD8^+$ T-lymphocytes.[77] However, MHC class I tetramers are limited to certain HLA class I alleles and do not select CD4+ virus-specific T-cells.

Thirdly, the interferon-γ (IFN-γ) capture assay has been developed. In this approach, PBMC are stimulated with viral peptide pools for a couple of hours. Virus-specific T cells become activated and produce IFN-γ. The IFN-γ secreting virus-specific cells are then labelled with magnetic beads and isolated via a magnetic column. This method enables the rapid enrichment of both $CD4^+$ and $CD8^+$ virus-specific cells. However, only a subset of virus-specific T cells secrete IFN-γ upon activation.[78;79] This method has been successfully used in the clinical setting to select T-cells specific for CMV, EBV and HAdV.[80-82]

Both tetramer-based and IFN-γ capture based techniques yield low numbers of virus-specific cells, which will proliferate *in vivo* upon antigenic stimulation after infusion in a patient with a viral reactivation. Transfused virus-specific T cells are detectable and provide protection for years after infusion. However, the production of virus-specific T cell products remains very labor-intensive and is only available in a small number of transplant centers. To address this problem, third party biobanks have been started in the United States and United Kingdom, in which virus-specific T cells from donors with common HLA-types are produced and cryopreserved, available for "of the shelve" use in HSCT recipients with a viral reactivation.[83]

3.3. Natural Killer cells

NK cells play a role in the innate immune defense through the direct lysis of virus-infected and malignant cells. Furthermore, they secrete cytokines and chemokines that initiate, enhance and regulate immune responses.[84-87] NK cells are a member of the large family of innate lymphoid cells (ILC). However, unlike the other ILC, NK cells are cytolytic and express perforin. They are classified in ILC group 1 based on their production of the inflammatory cytokines IFN-γ and TNF-α. They can be distinguished from other ILC by the expression of the transcription factor Eomesodermin (EOMES) and the cytolytic protein perforin.[88;89] In the last decade, the development of human NK cells from hematopoietic stem cells has been studied extensively. The first CD34+ stages of human NK cell development (stage 1 and 2) are observed in both bone marrow and secondary lymphoid tissues.[90;91] Subsequently, stage 3 NK cell development mainly occurs in secondary lymphoid tissues.[91-93] At this stage, NK cells acquire the expression of CD56 and express high levels of CD117 (c-kit) and CD127 (IL7Rα), showing close homology to type 3 ILCs.[88;91;94] Two distinct EOMES^{+}perforin^{+} NK cell populations are present in human peripheral blood: the minor CD56^{bright}CD16$^{+/-}$ (CD56^{bright}, stage 4) subset which constitutes 5-15% of NK cells, and the major CD56^{dim}CD16^{+} (CD56^{dim}, stage 5) subset (Figure 1.8).[87;95] CD56^{bright} NK cells are generally considered to be the precursors of CD56^{dim} NK cells.[91;96] For example, CD56^{bright} NK cells have longer telomeres and express low levels of stem cell factor receptor c-kit (CD117).[97;98] The expression of the inhibitory receptor NKG2A, expressed by all CD56^{bright} NK cells, is lost during the differentiation of CD56^{dim} NK cells, which ultimately acquire killer-immunoglobulin-receptors (KIRs) and the terminal differentiation marker CD57 (Figure 1.8).[99] Both subsets have their own functional profile; CD56^{bright} NK cells are mainly cytokine producers. Although they constitutively express perforin, they do not express granzyme B and require pre-activation to exert cytotoxicity (Figure 1.8). In contrast, CD56^{dim} NK cells constitutively express perforin and granzyme B and efficiently lyse target cells without prior stimulation.[87;95;100;101] However, both subsets can be cytotoxic and produce cytokines, after the appropriate *in vitro* stimulation.[100-105] Although the CD56^{dim} NK cells predominate in blood, the CD56^{bright} NK represent the majority of NK cells in both lymphoid and non-lymphoid tissues.[106-108]

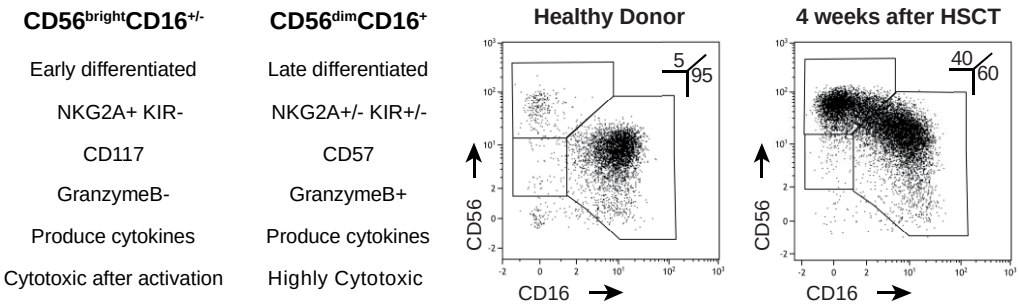

Figure 1.8. Circulating human NK cell subsets.
Hallmarks of CD56^{bright}CD16$^{+/-}$ and CD56^{dim}CD16^{+} NK cell subsets and their typical distribution in blood of healthy individuals and patients early after HSCT.

3.3.1 NK cell cytotoxicity, activation and tolerance

Unlike T cells, NK cells do not require prior activation to exert their killing function and are not restricted to MHC-class I presentation of viral antigens; rather, they recognize "missing self" (the down regulation of MHC molecules) and "induced or altered self" (the expression of stress-induced or virus-encoded ligands) on virus-infected or malignant cells.[87;109] The overall responsiveness of NK cells can be increased by monokines (*e.g.* IL2/IL12/IL15/IL18) produced by other immune cells like dendritic cells and monocytes (Figure 1.9).[87;110]The killing activity of NK cells is regulated by a balance of inhibitory and activating signals. In the presence of MHC class I molecules on a target-cell, inhibitory NK-cell receptors are triggered. This leads to the delivery of inhibitory signals via the intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) domain, preventing NK cell activation and lysis of the target-cell. When MHC class I molecules are downregulated, as in the case of tumor cells or virus- infected cells, no inhibitory signal is delivered. Instead, positive signals through activating receptors with an intracellular immunoreceptor tyrosine-based inhibitory motif (ITAM) dominate, which induces NK cell activation and lysis of target cells.[87;109;111]

Activating signals to the NK cell can be induced by the activating receptor NKG2D when it binds MHC class I-like ligands that are induced by virus infection and malignant transformation, but also by activating KIRs and the heterodimeric CD94-NKG2C receptors that recognize MHC-class I ligands, and by a number of other activating receptors (*e.g.* NKp30, NKp44, NKp46) for which the cellular ligands have yet to be identified.[87;109]

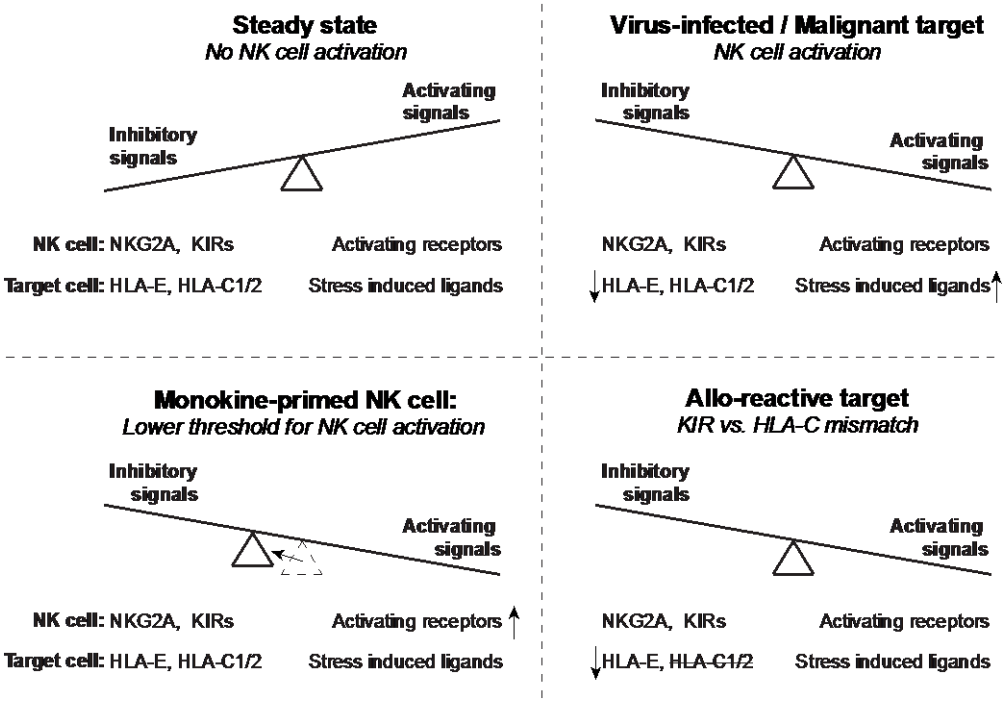

Figure 1.9. Principles of NK cell activation and tolerance.

Inhibitory NK cell receptors that recognize MHC-class I molecules include inhibitory killer immunoglobulin-like receptors (KIR) recognizing HLA-C molecules, and the inhibitory CD94-NKG2A heterodimeric receptors recognizing the non-classical HLA-E molecule. To prevent, the killing of healthy cells in the body (auto-reactivity), NK cells need to express at least one inhibitory receptor for MHC class I to be "licensed to kill": NK cells lacking an inhibitory receptor for self MHC are hyporesponsive.[102;111;112] The KIR expression repertoire, encoded on chromosome 19 is polymorphic amongst individuals. Two main groups of KIRs are recognized, the A and B haplotype. KIR A haplotypes recognize (and are inhibited by) HLA-C1 and the ligand for KIR B haplotypes is HLA-C2.[113] As KIRs and HLA-C are inherited independently, individuals can have NK cells expressing KIRs for which no ligand is available. These "uneducated" NK cells will become hyporesponsive.[102;112] In conclusion, the cytotoxicity of NK cells is the result of the balance between a) resting vs. activation/priming as mediated by monokines produced by other immune cells and b) inhibitory vs. activating signals by target cell-interaction (Figure 1.9).

3.3.2 Role of NK cells in immune responses

NK cells play a role in the control of viral load early during infection. NK cells contribute to the first line of immune defense through the lysis of virus-infected cells. Furthermore, NK cells play an important role in the initiation, enhancement and regulation of immune responses, directly through the secretion of cytokines and chemokines as well as indirectly via their interaction with other innate immune cells.[84-87;114-116] The best illustrations of the clinical relevance of NK cells are rare patients with isolated NK cell deficiencies, who are susceptible to herpesvirus infections.[117;118] CMV infection is associated with an increase in late differentiated $NKG2A^{-}$ $KIR^{+}NKG2C^{+}$ $CD56^{dim}$ NK cells.[119] In healthy individuals with primary EBV infections, an expansion of $CD56^{bright}$ and early differentiated $NKG2A^{+}KIR^{-}$ $CD56^{dim}$ NK cells has been described.[120;121]

Secondly, NK cells can function as anti-tumor effector cells via the elimination of malignancies with reduced MHC-class I expression that evade $CD8^{+}$ T cell-mediated control and of tumors with increased expression of activating NK cell receptor ligands.[87]

Thirdly, NK cell alloreactivity, based on the mismatch of KIR and KIR-ligands has been exploited in the setting of haplo-identical HSCT.[122-124] For example, when NK cells from a KIR A haplotype donor (tolerant for HLA-C1) are transplanted into a HLA C1 negative patient, no inhibitory signal is provided by the recipients hematopoietic cells, resulting in NK cell alloreactivity against HLA-C1 negative leukemic cells (Figure 1.8). Seminal studies have shown that the patients receiving a haplo-identical HSCT with NK cell alloreactivity in the graft vs. host direction have a reduced relapse rate and better overall survival.[122] In line with this, *in vitro* (monokine) activated donor derived NK cells have been exploited for the use as adoptive immunotherapy, especially in the haplo-identical HSCT setting, but also in the setting of primary malignancies susceptible for NK cell mediated cytotoxicity.[123;124]

3.3.3 NK cell reconstitution after HSCT

NK cells are the first lymphocytes that reconstitute after HSCT, reaching normal numbers within 3-4 week after HSCT.[33;125] Typically, the phenotype of NK cells after HSCT is skewed towards the ($NKG2A^+$) $CD56^{bright}$ NK cell phenotype (Figure 1.7). Because of the short life-time of NK cells and the phenotypic difference between the NK cells appearing early after HSCT and healthy donor NK cells, these NK cells are considered to reconstitute from stem cells in the graft. Various studies reported the existence of phenotypic and functional intermediate stages in the progression from $CD56^{bright}$ to $CD56^{dim}$ NK cells after HSCT. These studies mainly focused on CD16, CD27 or CD117, for which $CD56^{bright}$ NK cells have a bimodal expression profile.[100;126-128] The phenotype of $CD56^{dim}$ NK cells recovering after HSCT also differs from healthy donor $CD56^{dim}$ NK cells. The first $CD56^{dim}$ NK cells are mainly $NKG2A^+$ and KIR^-. $CD56^{dim}$ NK cells loose the expression of NKG2A and gain the expression of KIRs during the first year after HSCT, recapitulating NK cell differentiation.[99] In patients with CMV reactivation after HSCT, a specific expansion of $NKG2A^-KIR^+NKG2C^+$ $CD56^{dim}$ NK cells has been described.[129]

Contradicting data exist on the functional capacity of NK cells after HSCT. Various reports have described a normal cytotoxicity early after HSCT but a delayed recovery of IFN-γ production upon target cell recognition.[130-132] Other studies reported a reduced cytotoxicity of NK cells in the first months after HSCT.[133-135] A third group of studies showed that post-transplant NK cells display a reduced cytotoxicity against AML blasts expressing the NKG2A-ligand HLA-E, but have a normal killing of other target cells like K562 and EBV-LCL.[135-138] The relative hypofunction of NK cells after HSCT may be explained by induced tolerance because of the absence of self-KIRs for donor NK cells in the host. Another possible explanation for the reduced functionality of NK cells is the inhibition via NKG2A as most ($CD56^{bright}$ and $CD56^{dim}$) NK cells that reconstitute are $NKG2A^+$ after HSCT.

4 Objectives and outline of this thesis

Immune reconstitution is a major predictor of the outcome of viral complications after HSCT. A better understanding of immune reconstitution after HSCT and the interplay between different players of the immune system may provide tools to improve this immune reconstitution.

The **first part** (Chapter 2-4) of this thesis focusses on the relation between viral reactivations and T cell reconstitution. The importance of early T cell reconstitution to prevent and clear viral reactivations has been well established. However, the long term impact of viral reactivations on the composition of the immune system is largely unknown. In **Chapter 2**, we studied the impact of early CMV, EBV and HAdV infections on the composition and balance of the T cell compartment long term after HSCT. **Chapter 3** describes the development of a clinical grade method to select virus specific T cells for the restoration of T cell immunity for CMV, EBV and HAdV in one single procedure. For this, alternatives for the IFN- γ capture assay to select virus-specific T cells were explored, using the upregulation of various activation markers on virus-specific T cells upon peptide pool stimulation. **Chapter 4** addresses the effectiveness of pre-emptive Cidofovir therapy for HAdV infections after HSCT. To correct for the confounding effect of reconstitution of T cell immunity on the reduction of HAdV viral load during cidofovir treatment, we focused on patients lacking T cell reconstitution. We discovered that the reduction of HAdV load in the absence of T cells was always associated with an expansion of ($CD56^{bright}$) NK cells.

The **second part** (Chapter 5-7) of this thesis is focused on NK cells. In steady state conditions, the role of NK cells in anti-microbial immunity may be overshadowed by the presence of T cells. As NK cell reconstitution often precedes the recovery of T cells after HSCT, this creates a window of opportunity to study NK cells in the absence of T cells. **Chapter 5** addresses one of the restrictions of working with cryopreserved material. Whereas T cells are relatively resistant to cryopreservation, NK cells may display phenotypic changes which have to be accounted for when working with biobanked material. **Chapter 6**, describes the quantitative as well as phenotypic and functional reconstitution of $CD56^{bright}$ NK cells in patients with a rapid and a delayed T cell reconstitution to unveil changes in the NK cell compartment during transient T cell deficiency after HSCT. In **Chapter 7**, we studied the phenotype and homing potential of $CD56^{bright}$ NK cells in blood and lymphoid organs, which is the primary location for interactions between NK cells and other immune cells. We discovered a major tissue-resident NK cell population, which was further characterized. Also, the reconstitution of the different NK cell subsets in bone marrow after HSCT was studied.

The findings and implications of the work reported in this thesis are summarized in **Chapter 8** and discussed in **Chapter 9**.